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Bulk growth of *Neisseria gonorrhoeae* type 1 in a biphasic system

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SUMMARY A biphasic system for the bulk growth of *Neisseria gonorrhoeae* is described. It appears to combine the convenience of liquid media with the growth qualities of solid media. By using the agar in dialysis tubes rather than as slabs the surface-to-volume ratio was improved and harvesting made easier. The system is also useful in isolating gonococci from blood and joint fluids in patients with disseminated infection.

Introduction

Any major investigation of the antigenic or chemical make-up of *Neisseria gonorrhoeae* requires the growth of large quantities of bacteria. Although *N. gonorrhoeae* grows well on solid media, and such cultures are suitable for determining colony types, the preparation of solid cultures on any scale involves much manipulation with the consequent risk of contamination, and most of the organisms harvested will have been dead for many hours. The obvious alternative, growth in a fermenter, brings other problems. *N. gonorrhoeae* grows relatively slowly in liquid media so that large inocula are needed while even very few contaminants readily outgrow the neisseriae.

Gerhardt and Hedén (1960) pointed out that removal of inhibitory factors from the medium was as important as providing the right nutrients. By using a simple biphasic system in which liquid medium was shaken over the surface of dextrose starch agar in an Erlenmeyer flask they obtained good growth of *N. gonorrhoeae* even when the liquid phase was poor in nutrients. It is, of course, likely that all their strains of gonococci were of colony type 4.

We have investigated some of the factors in the biphasic system which affect the growth of *N. gonorrhoeae*, in particular the results of using the agar in dialysis tubes instead of as a single slab.

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Materials and method

BACTERIA

N. gonorrhoeae F62 is a descendant of the original type 1 strain described by Kellogg *et al* (1963). The type 4 strain, G22, was obtained by repeated subculture from an organism isolated in the diagnostic laboratory.

Both strains were stored in liquid nitrogen as described by Ward and Watt (1971). During use stock cultures were maintained by selective subculture on Difco GC base with supplements A and B. Colony types were determined by examination under combined incident and transmitted light as described by Kellogg *et al* (1963).

CULTURE METHODS

The bacteria were grown in 250-ml Erlenmeyer flasks by means of a biphasic system modified from that of Gerhardt and Hedén (1960). The liquid phase consisted of nutrient broth containing 15 g/l proteose-peptone (Difco no. 3) together with various supplements as desired.

Supplement A

Supplement A contained 20% dextrose, 0·25% α-glutamine (Sigma), and 0·002% co-carboxylase (Koch-Light) in distilled water and was sterilised by membrane filtration. It was added to GC base agar, 20 ml/1000 ml of agar, and to liquid medium, 4 ml/100 ml of broth.

Supplement B
Supplement B contained 0·5% ferric nitrate (Sigma) in distilled water and was sterilised by membrane filtration. It was added to GC base agar, 2 ml/1000 ml of agar, and to liquid medium, 0·4 ml/100 ml of broth.

SODIUM BICARBONATE
A 10% solution of sodium bicarbonate (BDH) in distilled water was prepared and sterilised by autoclaving at 10 lb/in² (69KPa) for 10 minutes (115°C) in a closed vessel. The solution was used at a final concentration of 0·012 ml/l.

The solid phase consisted of Difco's dextrose starch agar (DSA), 65 g/l, together with Bacto-agar, 10 g/l. Each flask contained 50 ml of agar in the form of a simple slab or as a sausage in 18/32″ dialysis tubing prepared for use by steaming in several changes of distilled water. Fifty millilitres of agar was contained in two lengths of approximately 150 cm each. The flasks, with agar and broth, were sterilised in the autoclave at 115°C for 10 minutes and allowed to cool. Supplements were sterilised by filtration and injected into the sterile flasks through the caps. Flasks were incubated overnight at 37°C before use to check for sterility.

INOCULUM
One or two colonies of type 1 or type 4 colonies were selectively subcultured on GC agar plates and incubated at 37°C in a carbon dioxide incubator (8% CO_2) for 18 hours. After the colony type had been checked the growth was scraped into warm proteose-peptone, pooled, and the optical density (λ = 700 nm) measured. The volume required was added to each flask with a syringe.

GROWTH
Flasks prepared and inoculated as above were put on a New Brunswick orbital shaker, oscillating at 100 cycles/min for six to eight hours at 37°C. At suitable intervals samples were taken to measure the optical density and to check the purity by Gram's stain and subculture. The colony type of subcultures was noted. Growth was harvested by pooling the liquid phase of all flasks and centrifuging at 6000 × *g* for 30 minutes.

DRY WEIGHT DETERMINATION
To determine the dry weight, 50 ml volumes of growth were centrifuged at 6000 × *g* for 30 minutes. The pellets were washed by resuspending in 50 ml of 0·1% formol water and recentrifuging at 6000 × *g* for 30 minutes. The resulting pellet was resuspended in 20 ml of 0·1% formol water, and 1 ml volumes in triplicate were placed in tared, preheated, aluminium foil caps. The dry weight was determined after heating at 80°C for 24 hours.

VIABLE COUNTS
From each flask 2 ml volumes were taken and ultrasonicated for 15 seconds (25 kHz). Before and after ultrasonication tenfold dilutions of the culture were made in warm proteose-peptone. From each dilution duplicate 0.1 ml samples were spread on GC agar plates and incubated at 37°C in a carbon dioxide incubator for 36 hours.

Results

GROWTH IN BASIC MEDIUM WITH AND WITHOUT SUPPLEMENTS AND AGAR
Over a six-hour period the type 1 strain did not grow in proteose-peptone alone. It grew poorly with either supplements or agar but well with both. The type 4 strain grew better than type 1 in the poor media. Supplement, though helpful, was not essential, almost maximum growth of type 4 being given by adding agar alone (Table 1). Type 1 strains were used for all subsequent experiments.

Table 1 *Growth of* Neisseria gonorrhoeae *in liquid and biphasic media with and without supplements*

Strain of N. gonorrhoeae	*Medium*	*Optical density* (700 *nm*)			
		Initially	*At six hours*	*Increase*	*Ratio*
F62 type 1	PPL alone	0·23	0·09	NG	—
	PPL + supplements A and B	0·14	0.28	0.14	2·0
	PPL + agar (1:1 ratio)	0·28	0·98	0·70	3·5
	PPL + agar + supplements A and B	0·16	1·28	1·12	8·0
G22 type 4	PPL alone	0·26	0·30	0·04	1·2
	PPL + supplements A and B	0·26	0·90	0·64	3·5
	PPL + agar (1:1 ratio)	0·21	1·65	1·44	7·9
	PPL + agar + supplements A and B	0·21	1·8	1·59	8·6

PPL = Proteose-peptone liquid
NG = No growth

EFFECT OF AGAR IN SLAB OR TUBES AND OF DIFFERENT AGAR/LIQUID RATIOS

With colonies of F62 (type 1) grown in biphasic medium with dialysis tubing an increase in the ratio of liquid medium to agar from 1:1 to 2:1 halved the density of growth reached at six hours so that the final yield was the same. An increase in the ratio to 3:1 gave only 80% of the expected yield. In flasks containing agar slabs there was a fall in yield to 82% even when the ratio was increased from 1:1 to only 2:1 (Table 2).

Table 2 *Growth of* N. gonorrhoeae *F62 type 1 in biphasic medium and effect of increasing liquid-to-solid ratio*

Agar	*Liquid phase (ml)*	*Optical density at six hours*	*Yield**	*Relative yield*
Slab (50 ml)	50	1·3	65	1
	100	0·53	53	0·82
	150	0·37	55	0·85
Tubes (2×25 ml)	50	1·5	75	1
	100	0·83	83	1·1
	150	0·44	66	0·8

*Optical density × volume

At all liquid-to-solid ratios the yield from flasks with tubes was greater than that from slabs. The agar in tubes had twice the surface-to-volume ratio of that in slabs, and the more efficient absorption thus obtained presumably accounts for the better yields. It was much easier to harvest bacteria from flasks with agar in tubes than from those with slabs. With larger volumes of liquid the slabs tended to disintegrate.

The appearance of the organisms grown differed markedly. Gram-stained films from flasks with agar in slabs showed large clumps of ragged-looking gonococci with no individual organisms and much debris. Films from agar-tube cultures showed smaller clumps of well-defined organisms, individual bacteria, and no debris.

With both slabs and tubes 95% of the colonies from sample subcultures were of type 1.

EFFECT OF INOCULUM SIZE

One of the problems in comparing different media was to ensure that they received equal inocula at the beginning. Over the periods of time studied the inoculum size significantly affected the final yield (Table 3).

In flasks receiving an inoculum which gave a starting optical density of 0·3 growth became logarithmic in under two hours. Growth was slower with inocula giving starting optical densities of 0·2 or 0·1.

Table 3 *Effect of inoculum size on yield of* N. gonorrhoeae *F62 type 1 in biphasic medium with and without added bicarbonate*

Sodium bicarbonate added	*Inoculum**	*Yield†*	*Increase (ratio)*
None	0·11	1·05	9·5
	0·20	1·38	6·9
	0·27	1·48	5·5
0·012 mol/l (final concentration)	0·12	1·48	12·3
	0·16	1·60	10·0
	0·25	1·85	7·4

*Initial optical density
†Optical density at six hours

From a practical point of view, although the ratio of yield to inoculum was greatest for the smallest inoculum, to obtain the best absolute yield the largest inoculum was necessary. With an initial optical density of 0·3 each flask with agar in tubes yielded 0·5–1 g wet weight of gonococci in six to eight hours.

EFFECT OF BICARBONATE

Because of the technical problems of supplying 10% carbon dioxide to shaking flasks, sodium bicarbonate was added to the medium in order to improve the yield as recommended by Talley and Baugh (1975).

The addition of sodium bicarbonate to a final concentration of 0·07 mol/l inhibited growth completely. At 0·036 mol/l and 0·012 mol/l concentrations the most striking effect was a prolongation of growth as determined by optical density for at least two to three hours beyond the time when bacteria in the control flask had entered the stationary phase.

Growth in biphasic flasks without sodium bicarbonate caused the pH of the medium to fall below 6·0, but the addition of sodium bicarbonate maintained the pH above 6·0. The inoculum effect described above was also found in the presence of sodium bicarbonate (Table 3).

Table 4 *Effect of added bicarbonate on yield of* N. gonorrhoeae *type F62 1 in biphasic culture*

	Optical density		*Weight (mg/ml) at six hours*		
	Initially	*At six hours*	*Wet*	*Dry*	*Wet/dry weight (%)*
Bicarbonate					
None	0·1	1·05	6·2	1·8	29
Added	0·12	1·30	10·3	1·8	17
Relative Yield	—	1·2	1·7	1	—
Bicarbonate					
None	0·2	1·45	5·8	2·0	34
Added	0·17	1·70	9·1	1·9	21
Relative Yield	—	1·2	1·6	1	—

The addition of bicarbonate to a final concentration of 0·012 mol/l appeared to increase the yield as measured by optical density (Table 3) and wet weight but not as measured by dry weight (Table 4). The viable count of F62 type 1 cultures grown over six hours increased approximately fivefold and was not significantly enhanced by the addition of sodium bicarbonate.

EFFECT OF ULTRASONICATION ON OPTICAL DENSITY AND VIABLE COUNT

Liquid cultures of type 1 colonies grew in macroscopic clumps unlike type 4 colonies, which grew in smooth suspension. Samples were taken from a log phase culture of F62 type 1 and ultrasonicated for 0, 15, 30, 45, and 60 seconds. Ultrasonication for 15 seconds produced a pronounced increase in optical density and a fourfold increase in viable count (Table 5). The Gram films showed disintegration of the clumps over 30 seconds. Both optical density and viable counts fell if ultrasonication was continued for more than 30 seconds.

Table 5 *Effect of ultrasonication on a log phase culture of* N. gonorrhoeae *F62 type* 1

Duration of ultrasonication (seconds)	*Optical density (700 nm)*	*cfu/ml* $\times 10^8$
0	0·78	1·03
15	1·38	3·94
30	1·48	3·6
45	1·35	3·2
60	1·35	2·7

Discussion

N. gonorrhoeae grew readily in a biphasic system, and we found, as have others (Sparling, 1966; Jephcott, 1972; Hart and Goldberg, 1975), that type 1 strains could be well maintained. Although Hart and Goldberg obtained viable counts of 10^{10} colony-forming units (cfu) per ml neither we nor Jephcott obtained much more than 3×10^8 cfu/ml. The significance of such comparisons is doubtful in view of the wide variations in clump sizes found in this system. More meaningful figures for opacity measurements and viable counts are obtained if suspensions were first given mild ultrasonic treatment. The yields of bacteria in terms of weight are good enough to make the system of practical use. Unfortunately neither Jephcott (1972) nor Hart and Goldberg (1975) gave weight yields for comparison.

The use of agar in dialysis tubes instead of as slabs made harvesting much easier and gave larger yields of healthier-looking bacteria. The effect is presumably due to the greater surface-to-volume ratio obtainable with tubes. The apparent increase in yield with the addition of sodium bicarbonate is largely due to the increased water content of the bacteria. Other possible effects of bicarbonate on the chemical composition of the bacteria were not determined.

Yield ratios were greatest with small inocula, but absolute yields increased with increasing inoculum size. The results suggest that good yields depend on rapid growth over a short period and that loss by death and lysis becomes a significant factor after a few hours.

Further improvement in yields should be possible, probably more by improving the efficiency of absorption than by adding further nutrients, although both should be tried.

The effectiveness of the biphasic system in growing gonococci suggested that it might have a place in clinical bacteriology. Samples of blood and joint fluid from patients with suspected disseminated gonococcal infection were cultured in bottles containing 50 ml of liquid medium and 25 ml of agar. Preliminary results are encouraging when compared with isolation rates obtained by conventional methods.

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